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Expression, purification and characterization of recombinant human proinsulin

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Abstract We have recently developed a method to produce native human proinsulin using a bacterial expression system. A proinsulin fusion protein was recovered from inclusion bodies and cleaved using cyanogen bromide. The released proinsulin polypeptide was *S*-sulfonated and purified by anion exchange chromatography. Following refolding, proinsulin was purified by reversed-phase high-performance liquid chromatography. Combined peptide mapping and mass spectrometric analysis indicated that the proinsulin contained the correct disulfide bridging pattern. This proinsulin will be used to study the specificity of the furin/PC family of converting enzymes by using it as a substrate in a recently developed assay.

Key words: Proinsulin; Expression; Recombinant

1. Introduction

Many biologically active peptides and proteins are produced as inactive or less active precursors which must undergo enzymatic cleavage or processing to obtain their full potency [1–3]. The first hormone shown to be produced via a precursor was proinsulin [4]. Recently, proinsulin has been shown to be processed by members of the furin/PC family of propeptide converting enzymes (PCEs). PC1 cleaves proinsulin at a pair of basic amino acids located between the B chain and C-peptide, while PC2 cleaves between the C-peptide and A chain [5,6].

To examine further the specificity of these PCEs, we need to be able to routinely produce milligram quantities of native proinsulin for kinetic studies. Bacterial expression systems have been used to produce many mammalian proteins and peptides [7,8]. The expressed proteins have been used in a variety of different applications such as antigen production, structural studies and biochemical or cell biological assays. While bacteria have some disadvantages, including the absence of intracellular sorting and packaging mechanisms, they do have many advantages over mammalian expression systems. These advantages include ease of maintaining bacterial cultures, high level expression of recombinant proteins, and the simplicity of *E. coli* genetics. In recent years, a number of attempts have been made to express both insulin and proinsulin in bacteria [9–14]. These attempts have included both expression of the B and A chains separately, and the expression of intact proinsulin. Many of these attempts failed because of N-terminal proteolytic degradation of the recombinant protein within bacterial cells [11]. Other groups have successfully produced proinsulin, but their published reports have generally been incomplete, presumably to protect proprietary interests [9,12–14].

We have developed a method which allows us to express proinsulin in bacteria by combining the procedures of both published and partially described, proprietary methods. In short, a fusion protein composed of an N-terminal polyhistidine affinity tag, a methionine residue, and the human proinsulin sequence was constructed in a bacterial expression vector. Following expression, proinsulin was cleaved from the polyhistidine tail, purified, and then refolded into its native state. The recombinant proinsulin was analyzed by mass spectrometry and peptide mapping to verify its identity and the formation of the correct disulfide bridging pattern. This recombinant proinsulin can be used in future studies for analyzing the specificity of PC1 and PC2.

2. Materials and methods

Human proinsulin was the gift of Eli Lilly and Co. (Indianapolis, IN). The human cDNA library was obtained from Clontech (Palo Alto, CA), and the bacterial expression system was from Qiagen (Chatsworth, CA). Synthetic oligonucleotides were synthesized, and automated DNA sequencing was performed in the molecular biology core facility of Creighton University. Competent XL2-Blue MRF′ bacteria were obtained from Stratagene (LaJolla, CA), and restriction endonucleases were from New England Biolabs (Beverly, MA). *Staphylococcus aureus* V8 protease was obtained from Sigma Chemical Co. (St. Louis, MO).

2.1. PCR amplification of human proinsulin

Oligonucleotide primers were designed with specific restriction sites for *Bam*HI and *Sal*I (5′ and 3′, respectively) which allowed for directional cloning into the vector pQE-31 (Qiagen). In addition, the primer contained the DNA sequence coding for the amino acid methionine immediately 5′ of the proinsulin coding sequence.

A human pancreas cDNA library (Clontech) was used as a template, together with the specific primers, in the polymerase chain reaction (PCR) amplification of the human proinsulin coding sequence. The amplified DNA was analyzed by agarose (1.0%) gel electrophoresis to check for purity and proper size of the amplified product. The cDNA was ethanol precipitated and then resuspended in a small volume of dH_2O. The cDNA concentration was determined using Hoescht dye fluorescence.

The PCR product and pQE-31 expression vector were cut with the appropriate endonucleases, *Bam*HI and *Sal*I. The vector was dephosphorylated by treatment with calf intestinal phosphatase (CIP), and both the dephosphorylated vector and cut PCR fragment were ethanol precipitated and quantitated as described above. The PCR product was then cloned into the pQE-31 expression vector using Ready-to-go T4 DNA ligase (Pharmacia Biotech). The ligations were performed at 12°C for 15 min in a sonicator waterbath.

The newly constructed plasmid, hPI/pQE-31, was transformed into XL2-Blue MRF′ ultracompetent cells (Stratagene). Transformed bacteria were grown at 37°C on LB/ampicillin plates, and individual colonies were picked and grown overnight at 37°C in 4 ml LB broth containing 100 μg/ml ampicillin. The bacteria were collected by centrifugation, and plasmid DNA was purified using the Wizard DNA purification system (Promega). A portion of the plasmid DNA was

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digested with the endonucleases *Bam*HI and *Sal*I, and loaded onto a 0.8% agarose gel to verify the presence of the proinsulin cDNA insert. Integrity of the insert DNA was verified by both automated and manual DNA sequencing, and sequence alignment was performed using Geneworks (version 2.3) software on a Macintosh computer.

2.2. Expression screening for proinsulin

A culture of hPI/pQE-31 containing bacteria was grown in 5 ml LB/ampicillin (100 μg/ml) overnight and 1 ml of the overnight culture was used to inoculate 9 ml of prewarmed LB/ampicillin. The culture was grown in a shaking incubator at 37°C until A_{600} reached approx. 0.7–0.8. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and the culture was grown for an additional 3 h. Cells were harvested by centrifugation at 5000×*g* for 10 min at 4°C and the bacterial pellet was resuspended in 2 ml of ice cold 100 mM Tris-HCl, pH 7.3. Cellular membranes were disrupted by probe sonication (two cycles of 5 s on, 5 s off) and insoluble proteins were obtained by centrifugation at 15000×*g* for 10 min at 4°C. The pellet was resuspended in 1 ml of 20 mM Tris-HCl, pH 8.2, 7 M urea and shaken at room temperature for 30 min. Urea insoluble material was removed by centrifugation at 15000×*g* for 10 min at 4°C. Total protein was determined by the Bradford method (Bio-Rad) [15]. Protein expression was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by loading 10 μg of total protein, from the urea soluble fraction, onto a 15% polyacrylamide gel. Proteins were stained with Coomassie blue for 15 min and individual proteins were visualized by destaining with 0.5 M NaCl.

2.3. Small scale protein expression

A 1 l culture of hPI/pQE-31 containing bacteria was grown and protein expression induced as described above. Cells were harvested by centrifugation at 5000×*g* for 10 min at 4°C, and the bacterial pellets were resuspended in 5 ml/g (wet wt. bacteria) of 100 mM Tris-HCl, pH 7.3. Lysozyme was added at a concentration of 1 mg/ml and placed on a rotating shaker at room temperature for 15 min. The lysate was subjected to probe sonication for two cycles of 30 s on/30 s off at 4°C. Cellular debris was removed by centrifugation at 1000×*g* for 5 min at 4°C. The supernatant was retained and centrifuged again at 27000×*g* for 15 min at 10°C to pellet the inclusion bodies. The supernatant was discarded and the pellet resuspended in 1 ml/g (original wt. bacteria) of dH_2O, aliquoted into 1.5 ml microcentrifuge tubes as 1 ml fractions, and then centrifuged at 16000×*g* for 5 min at 4°C. The pellets were individually washed with 1 ml of 100 mM Tris-HCl, pH 8.5, 1 M urea, 1.0% Triton X-100, and again with 100 mM Tris-HCl, pH 8.5, 2 M urea, 2.0% Triton X-100. The pellets were resuspended in 1 ml of dH_2O and transferred to a preweighed 30 ml Corex centrifuge tube. The sample was centrifuged at 15000×*g* for 5 min at 4°C, and the pellet was resuspended in 10 ml/g (wet wt. pellet) of 70% formic acid. Cyanogen bromide was added to a final concentration of 400 mM and the sample was incubated at room temperature in the dark for 16 h. The reaction was stopped by transferring the sample to a round bottom flask and removing the solvent by rotary evaporation at 50°C. The residue was resuspended in 20 ml/g (wet wt. pellet) of dH_2O, shell frozen in a dry ice ethanol bath, and then lyophilized. The lyophilized protein was dissolved in 20 ml/g (wet wt. pellet) of 500 mM Tris-HCl, pH 8.2, 7 M urea. Oxidative sulfitolysis was performed by adding sodium sulfite and sodium tetrathionate to final concentrations of 100 and 10 mM, respectively, and incubating at room temperature for 3 h. This reaction was stopped by freezing on dry ice.

2.4. Purification and refolding of human proinsulin

The *S*-sulfonated material was applied to a 2 ml bed of Sephadex G-25 equilibrated in 20 mM Tris-HCl, pH 8.2, 7 M urea, and then washed with 9 vols. of 7 M urea. The collected fraction was applied to a Pharmacia Mono Q HR 5/5 column equilibrated in 20 mM Tris-HCl, pH 8.2, 7 M urea at a flow rate of 1 ml/min. A linear gradient leading to a final concentration of 0.5 M NaCl was used to elute the bound material. 2 min (2 ml) fractions were collected during the gradient, and protein concentration in each fraction was determined. Purity and molecular mass of fractions were estimated by Tricine SDS-PAGE [16], where Tricine is used as the trailing ion to allow better resolution of peptides in the range of 1–1000 kDa. Appropriate fractions were pooled and applied to a 1.6×20 cm column of Sephadex G-25 (superfine) equilibrated in 5 mM ammonium acetate pH 6.8. The sample was collected based on UV absorbance and freeze-dried. The partially purified *S*-sulfonated material was resuspended in 50 mM glycine/NaOH, pH 10.5 at a final concentration of 2 mg/ml. β-mercaptoethanol was added at a ratio of 1.5 mol per mol of cysteine *S*-sulfonate and the sample was stirred at 4°C in an open container for 16 h. The sample was then analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Vydac C_4 column (2.2×150 mm) equilibrated in 4% acetonitrile and 0.1% TFA. Adsorbed peptides were eluted with a linear gradient of increasing acetonitrile concentration (0.88% per min up to a maximum of 48%). The remaining refolded proinsulin sample was centrifuged for 5 min at 16000×*g* to remove insoluble material, and loaded onto a semi-preparative Vydac C_4 column (10×250 mm). The bound material was eluted as described above, and the proinsulin was collected and lyophilized.

2.5. Analysis and characterization

The purified expressed proinsulin was subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis (Protein and Carbohydrate Structure Facility, University of Michigan), using proinsulin from Eli Lilly as both an internal and external standard. To determine if the disulfide bridges had formed correctly, a

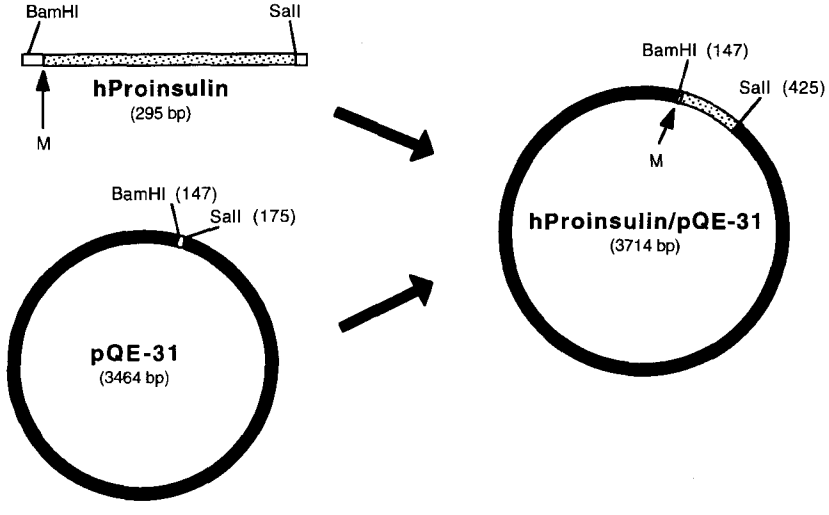

Fig. 1. Diagram representing the construction of the proinsulin expression plasmid. The PCR amplified Met-proinsulin cDNA containing *Bam*HI and *Sal*I restriction sites, and the expression vector pQE-31 containing the same restriction sites are shown. The final product of the ligation is the expression plasmid containing the Met-proinsulin cDNA.

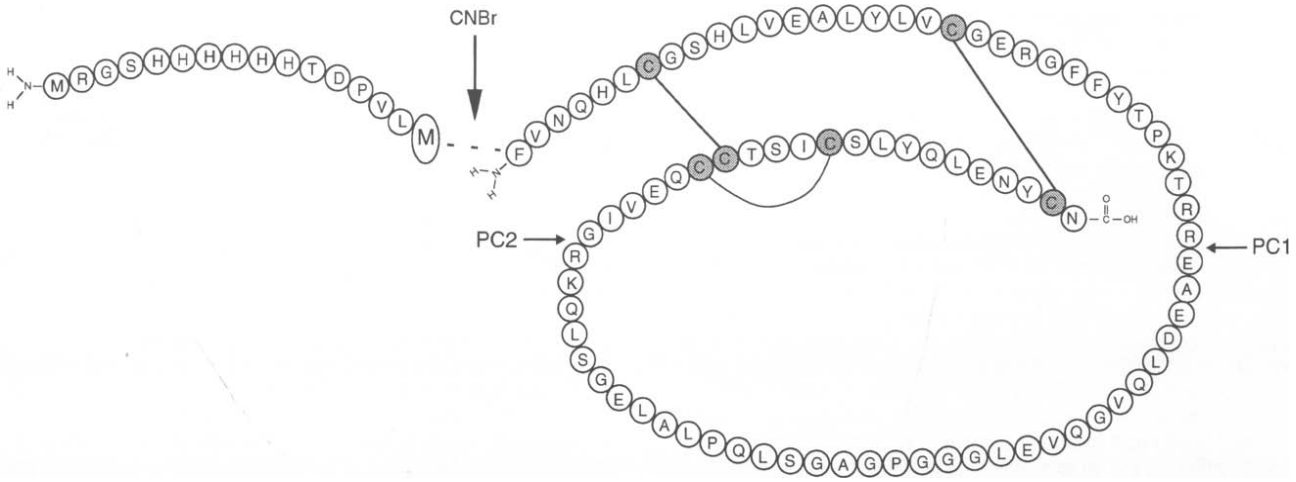

Fig. 2. Diagrammatic representation of the proinsulin fusion protein. The polyhistidine tag is cleaved from proinsulin by CNBr. Note that this diagrammatic representation depicts proinsulin with correctly linked disulfide bonds. These bonds have likely not formed when proinsulin is still attached to the polyhistidine tag. The sites of normal post-translational processing in islet beta cells are also indicated.

proteolytic digestion was performed using *Staphylococcus aureus* protease V8. 5 μg of both the expressed proinsulin and Eli Lilly's proinsulin were lyophilized and resuspended in 50 μl of 250 mM $NaPO_4$, pH 7.8. Protease V8 was added at a ratio of 1:50 (w/w) in experimental samples and no enzyme was added to the controls. All samples were then incubated overnight at 37°C, the reactions were stopped by freezing on dry ice, and samples were stored at −20°C until analyzed. The samples were analyzed by RP-HPLC using a Vydac C_4 column (2.2×150 mm) equilibrated in 4% acetonitrile and 0.1% TFA. Bound material was eluted using a linear gradient of increasing acetonitrile concentration (0.88% per min up to a maximum of 48%).

3. Results

3.1. Design of the bacterial expression system

The human proinsulin cDNA sequence was PCR amplified from a human pancreas cDNA library using a 5′ primer encoding the amino acid methionine immediately upstream of the proinsulin coding sequence (Fig. 1). A 1% agarose gel was run to verify the presence of the proper size PCR product, and only a single band was present with an estimated size of approx. 300 base pairs (data not shown). Following restriction enzyme digestion, the insert and vector were ligated together at a molar ratio of 5:1. The presence of the insert within the plasmid was verified by restriction mapping. Sequence integrity was determined by both automated and manual DNA sequencing (data not shown).

3.2. Protein production

The expression vector was transformed into an *E. coli* host and expressed intracellularly. The expressed proinsulin fusion protein was found to accumulate as insoluble inclusion bodies, as determined by SDS-PAGE analysis of both the soluble and insoluble cellular lysate fractions (data not shown). The insoluble inclusion bodies were washed with urea and Triton X-100 and the total wet weight of the obtained inclusion bodies was approx. 200 mg/l of bacterial culture. This material was solubilized in 70% formic acid and the

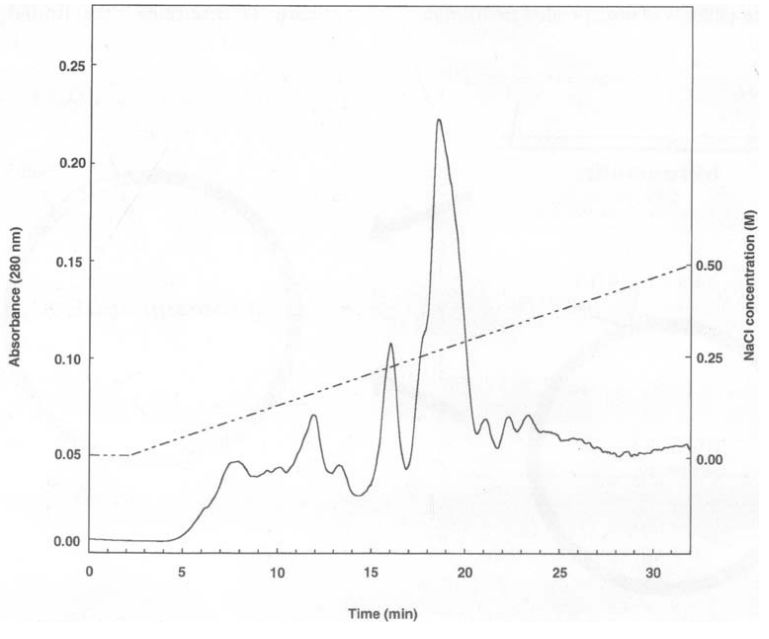

Fig. 3. Anion exchange chromatography of *S*-sulfonated proinsulin. This peptide was purified using a Mono Q HR 5/5 column (Pharmacia) on a BioLogic Work Station (Bio Rad). Fractions were analyzed and showed that the proinsulin is contained within the major peak.

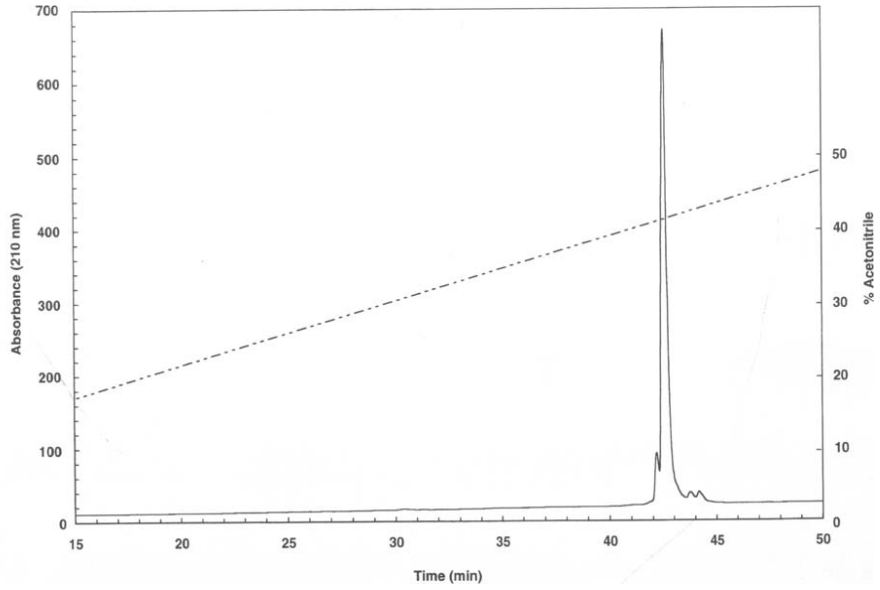

Fig. 4. Analytical RP-HPLC of refolded human proinsulin. The peptide was analyzed using a Vydac narrow bore column (2.2×150 mm) on a Hewlett Packard 1090 LC.

proinsulin was separated from the N-terminal polyhistidine tag by cyanogen bromide cleavage (Fig. 2). After chemical cleavage, the material was resuspended in 7 M urea, subjected to oxidative sulfitolysis [17,18], and the *S*-sulfonated proinsulin was purified by anion exchange chromatography (Fig. 3). Approx. 5–8 mg of partially purified, *S*-sulfonated proinsulin was normally obtained from 1 l of cultured bacteria. Following buffer exchange the proinsulin was simultaneously folded and re-oxidized at an approximate protein concentration of 2 mg/ml. The renatured proinsulin was purified by semi-preparative RP-HPLC, lyophilized, and its purity was checked by both analytical RP-HPLC and Tricine SDS-PAGE. Analytical RP-HPLC indicated the presence of a single major component at 90% purity (Fig. 4). Final yield of properly refolded proinsulin was 1–2 mg per l of bacterial culture.

To confirm the identity of this peptide, proinsulin from Eli Lilly was subjected to chromatography under identical conditions and it eluted with the same retention time. Samples of our expressed proinsulin and Eli Lilly proinsulin were then mixed together and subjected to the same chromatography procedure. The peptides eluted as a single peak (data not shown). Tricine SDS-PAGE was then used as an additional method for testing sample purity and checking its molecular mass (Fig. 5). The stained band seen in lane 3 represents the proinsulin fusion protein with an approximate molecular mass of 11 000 Da, while lane 5 shows purified recombinant human proinsulin with an estimated molecular mass of 9000 Da. The expressed proinsulin appears to be highly purified and co-migrates with Eli Lilly's proinsulin (lane 6).

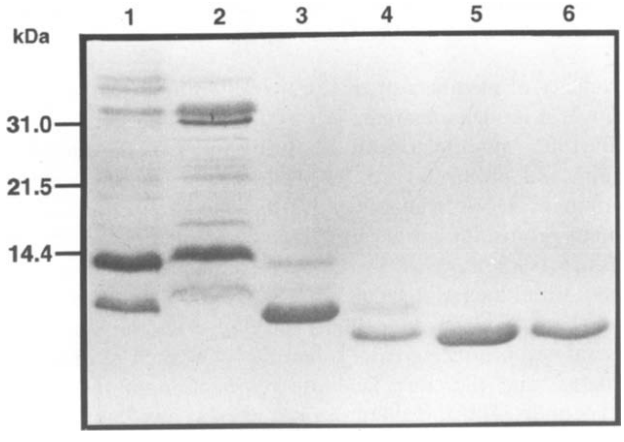

Fig. 5. SDS-PAGE analysis of expressed and purified proinsulin. Approx. 10–12 μg of protein was loaded in lanes 1–3 and approx. 5–8 μg of protein was loaded in lanes 4–6 of a 16.5% Tricine SDS-polyacrylamide gel. Lanes: 1, post sonicate proteins; 2, combined urea/Triton X-100 washes; 3, washed inclusion bodies; 4, cyanogen bromide cleaved fusion protein; 5, refolded RP-HPLC purified proinsulin; 6, proinsulin from Eli Lilly and Co. Protein bands were visualized by staining with Coomassie blue.

3.3. Protein analysis

MALDI-TOF analysis of the recombinant proinsulin verified the correct molecular mass of our expressed proinsulin using proinsulin provided by Eli Lilly as an external standard (see Table 1). Following this, both our refolded proinsulin and the Eli Lilly sample were combined and analyzed in a separate MALDI-TOF experiment. Results from this experiment verified that both proinsulin samples have the same molecular mass.

To test further whether the recombinant proinsulin had refolded properly, a proteolytic digestion was performed using *S. aureus* protease V8. The proteolytic digest was analyzed by RP-HPLC, again using proinsulin provided by Eli Lilly as a standard. RP-HPLC analysis indicated that V8 digestion of both the recombinant and Lilly proinsulin produced identical chromatograms (Fig. 6). The peptides generated from our expressed proinsulin were collected and MALDI-TOF analysis of the individual fractions was consistent with the formation of the correct disulfide bonds (see Table 1 and Fig. 7).

4. Discussion

For more then a century, researchers have studied the roles and actions of insulin. In 1967, Steiner and colleagues discovered a precursor to insulin which they named proinsulin [4].

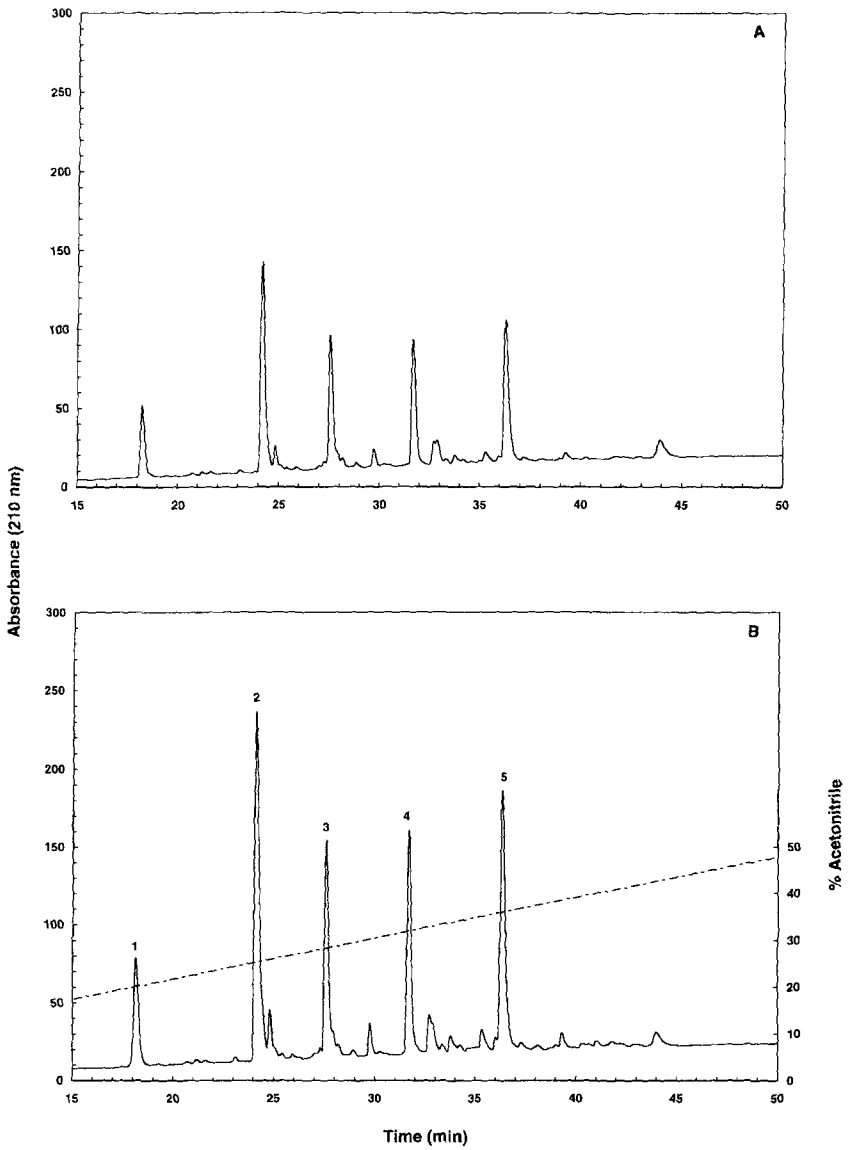

Fig. 6. Peptide mapping of proinsulin. RP-HPLC of Eli Lilly proinsulin (A) and refolded expressed proinsulin (B) which have been cleaved with *Staphylococcus aureus* protease V8. Molecular weight of the fragments was determined by MALDI-TOF. Peak 1, fragment 7; peak 2, fragment 3; peak 3, fragment 2, and fragment 1 shortened; peak 4, fragment 6; peak 5, fragment 1 (see Fig. 7).

Human proinsulin is translated as a single polypeptide chain and following removal of the signal sequence, it consists of 86 amino acids crosslinked by three disulfide bridges. Within the secretory pathway, fully bioactive insulin is generated by cleavage of proinsulin at two different sites, each of which have two adjacent basic amino acid residues [19–21]. This results in the separation of the cross-linked A and B chains from the C-peptide.

Recently, PC1 and PC2 have been implicated as the two members of the furin/PC family involved in the processing and activation of proinsulin [5,6,22]. The newly discovered furin/PC family of serine proteases, believed responsible for propeptide conversion, currently consists of seven members, including furin [23,24], PACE4 (paired basic amino acid converting enzyme [25,26], PC1 (also called PC3) [27,28], PC2 [29,30], PC4 [31], PC6 (also called PC5) [32,33], and PC7 [34]. The sequences of the mammalian PCs are homologous to the bacterial subtilisins, but exhibit markedly different substrate specificity. Our laboratory is interested in examining the specificity of members of this protease family by using proinsulin as a model substrate.

In 1981, investigators at Eli Lilly and Co. reported that insulin and proinsulin can be produced in bacteria using recombinant DNA technology [9,10]. Bacterial expression of human proinsulin has several advantages over eukaryotic expression (such as yeast, insect, or mammalian expression systems). First, bacterial genetics are fairly simple in comparison with that of eukaryotic systems. Second, bacterial cultures in general will produce greater quantities of desired recombinant proteins, and therefore facilitate purification of the desired polypeptide. Third, bacteria do not express any known forms of yeast *kex2* [35] or mammalian convertases, which could possibly result in the processing of the majority of the expressed proinsulin before purification. However, the procedures used by Eli Lilly and Co. have never been fully described. In addition, previous attempts by other investigators had suggested that bacterial expression of proinsulin resulted in very poor yields due to N-terminal degrada-

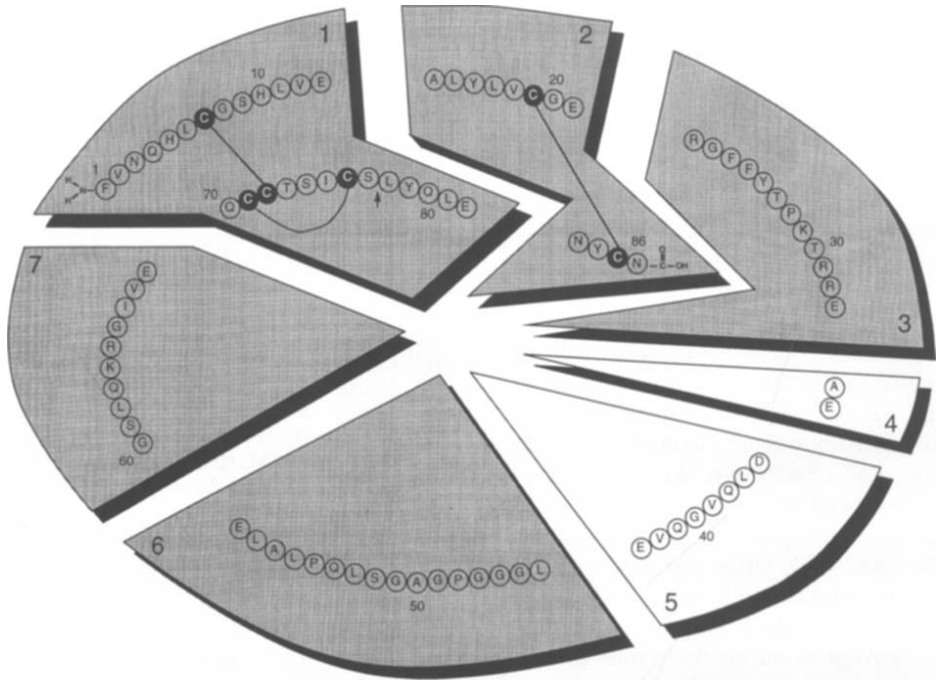

Fig. 7. Diagrammatic representation of protease V8 mapping of proinsulin. The fractions in white represent fragments that were not detected by combined RP-HPLC/MALDI-TOF. The arrow within fragment one indicates an additional cleavage site which was identified by MALDI-TOF analysis (fragment 1 shortened).

tion [11]. We have therefore developed a procedure, based upon the work of investigators at Eli Lilly and elsewhere [9,13,14] to produce proinsulin.

We designed a fusion protein expression system consisting of an N-terminal polyhistidine tail connected to the human proinsulin via a methionine residue. The polyhistidine tail was added to help stabilize the expressed peptide and prevent N-terminal degradation, and could be used for affinity purification if needed. The expressed fusion protein was found within inclusion bodies, which are believed to be non-crystalline, amorphous structures, composed of aggregates of partially folded proteins [36]. Recovery of washed inclusion bodies is usually performed by low speed centrifugation and repeated washing with detergents and denaturants. These washes help remove contaminants such as unlysed whole cells, peptidoglycan cell walls and soluble proteins [37] which may interfere with purification. The inclusion bodies were then solubilized in 70% formic acid and the polyhistidine tail cleaved from the proinsulin using cyanogen bromide. The proinsulin was then subjected to oxidative sulfitolysis which results in the formation of cysteine *S*-sulfonate derivatives [9]. This derivatization protects the sulfhydryl group from reaction with other thiols and/or irreversible oxidation, and in addition, pairs of *S*-sulfocysteines can be converted to cystine by simple air oxidation. The rate of this reaction can be altered, and mismatched disulfides can be broken (to allow proper refolding) by the addition of sulfhydryl compounds. An additional advantage is that this highly charged version of proinsulin can be partially purified by anion exchange chromatography. Following removal of the urea and buffer salts, the protein is then refolded in the presence of low concentrations of β-mercaptoethanol [9]. The refolded proinsulin was then purified by semi-preparative RP-HPLC and analysed by analytical RP-HPLC and Tricine SDS-PAGE. It appears that our expressed form of proinsulin is greater than 90% pure.

Table 1
Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis of protease V8 peptide mapping of recombinant human proinsulin

	Mass spectrometry	
	Actual	Theoretical
Proinsulin		
Recombinant proinsulin	9390.8	9394.8
Eli Lilly proinsulin	9389.6	9394.8
Combined	9390.7	9394.8
V8 generated peptides		
Fragment 1	2953.5	2955.4
Fragment 1 (shortened)	2308.5	2308.7
Fragment 2	1377.7	1379.6
Fragment 3	1558.5	1557.8
Fragment 6	1435.8	1436.6
Fragment 7	1087.7	1086.3

Eli Lilly has previously conducted extensive testing on their recombinant human proinsulin to assure proper refolding of the disulfide bonds [13]. Additional information was obtained by testing isoforms of mismatched disulfide bonded human insulin, which apparently are separable from native insulin using RP-HPLC [14]. We therefore compared our recombinant proinsulin with the proinsulin provided by Eli Lilly. Initially, we checked that our proinsulin has the same molecular mass as the Eli Lilly proinsulin. To assure that proper refolding and disulfide bond formation of our proinsulin had occurred, V8 protease was then used for peptide mapping. Under the conditions used, this protease hydrolyzes peptide bonds at the carboxyl side of glutamyl and aspartyl residues. After V8 digestion of proinsulin, there should be seven fragments created by eight potential cleavage sites (Fig. 7). Combined RP-HPLC and mass spectrometric analysis identified five of the seven fragments within a mass error of 0.1%.

The two smallest fragments (unshaded wedges, Fig. 7) were not detected. Fragment one has a theoretical mass of 2973.4 Da, but due to the presence of an N-terminal glutamine, cyclization occurs with a reduction in mass of 18.0 Da. Johnson [14] also reported that V8 is able to cleave between Ser-77 and Leu-78 within fragment one (see Fig. 7). This fragment apparently coeluted with fragment two during RP-HPLC, and was also identified by mass spectrometry. Based on the results from these analyses, we believe that we have obtained authentic human proinsulin in a form suitable for use in our propeptide conversion assay.

Because proinsulin contains three disulfide bonds, and the presence of these disulfide bonds can be confirmed by structural characterization, we feel that the three-dimensional structure of proinsulin in vitro will approximate the three-dimensional structure of proinsulin in vivo. We are, therefore, interested in using our expressed proinsulin as a substrate for examining the three-dimensional specificity of the propeptide converting enzymes PC1 and PC2. Using this model system, and having the ability to produce milligrams quantities of proinsulin, we should now be able to determine the exact site of PCE-mediated cleavage based on the results of mass spectrometric analysis of the reaction products. In addition, because we can produce modified expression plasmids in our laboratory, we should be able to alter the structure of the proinsulin using the techniques of DNA mutagenesis. This will allow us to modify the three-dimensional interactions between the enzyme and its propeptide substrate. Eventually, we hope to be able to predict, or possibly change, the specificity of potential processing sites recognized by individual members of the furin/PC family of enzymes.

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